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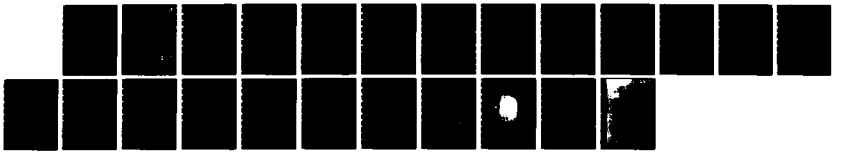

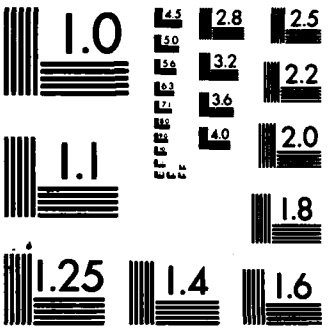

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A MICROAFFINITY ASSAY FOR PLASMA FIBRONECTIN

by

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ABSTRACT

The quantitation of fibronectin in human plasma has been based exclusively on immunochemical methods to-date. An alternate method is described which utilizes gelatin-coated Sepharose 4B beads to bind the fibronectin present in plasma, and by a series of washings aided by centrifugation to remove the unbound material. Elution of the protein with 6 M urea followed by a colorimetric reaction with the Bio-Rad protein dye reagent (Coomassie Blue G-250) produces a quantitative estimation of fibronectin in plasma. Twenty-two human plasma samples from healthy volunteers were analyzed by the microaffinity assay and by a commercially available immunoturbidimetric assay kit. There was a poor correlation ($r = 0.5032$) between the two assays. The concentration of fibronectin in plasma was 356 ± 87 μg/ml by the microaffinity assay, and 270 ± 57 μg/ml by the immunoturbidimetric method.

INTRODUCTION

Fibronectin is a normal circulating plasma protein which among its various biological properties (see References 1-3) has also been assigned the role of major opsonic fraction for the reticuloendothelial system (4). Therefore, there is an interest in methods of assaying levels of fibronectin in human and animal plasma which may provide additional data to further elucidate its involvement in hemorrhagic and septic shock resulting from trauma, or burns (5,6).

Several methods exist for the quantitation of human plasma fibronectin utilizing immunological principles. These include crossed immunoelectrophoresis (7), radioimmunoassay (8), solid-phase radioimmunoassay (9,10), enzyme-linked immunoassay (11,12), and immunoturbidimetry (13,14). Review of the pertinent literature shows that there is no general agreement among the various methods on the level of fibronectin in normal human plasma. Typical mean values range from 270 to 557 μg/ml depending on the assay used.

MATERIALS AND METHODS

Fibronectin Purification. Freshly prepared human plasma using CPD (citrate-phosphate-dextrose) as anticoagulant was employed as the source of fibronectin. The purification procedure was a modification of the method of Vuento and Vaheri (15) which involves affinity chromatography with Sepharose 4B immobilized gelatin and elution with arginine under non-denaturing conditions.

The plasma sample (118 ml) was applied on a chromatographic column packed with 147 ml gelatin-coated Sepharose 4B beads (see below) equilibrated with phosphate-buffered saline (PBS)/citrate buffer, pH 7.4. The PBS/citrate buffer consists of 1.2 g Na_2HPO_4, 0.22 g $NaH_2PO_4 \cdot H_2O$, 8.5 g NaCl, 2.94 g sodium citrate $\cdot$ 2 H_2O, and 0.2 g sodium azide, adjusted to pH 7.4 and made up to 1000 ml with water. The unbound material was eluted with the PBS/citrate buffer until the UV absorbance at 280 nm reached a baseline on the recorder. At this time 50 ml of 1 mM iodoacetamide in PBS/citrate buffer solution was passed through the column to block the sulfhydryl groups of fibronectin and, therefore, improve solubility and storage life (16). Subsequently, the column was washed with PBS/citrate buffer and the bound fibronectin was eluted with 1 M arginine in the same buffer. The UV absorbing fractions were collected, pooled and dialyzed in sequence vs. the PBS buffer without the citrate and vs. water, exhaustively. The dialyzed material was freeze-dried and checked for purity by SDS polyacrylamide gel electrophoresis (17) in 5% T and 10% T gels. The fibronectin preparation thus obtained was more than 95% pure.

Preparation of Gelatin-Coated Beads. The gelatin-coated Sepharose 4B beads were prepared by a minor modification of the method of Vuento and Vaheri (15).

Cyanogen bromide activated Sepharose-4B (CNBr-4B) (Sigma) (45 g) was washed with nine liters of 10^{-3} N HCl. A gelatin solution (1 mg/ml) was prepared by dissolving 0.225 g gelatin Type I (Sigma) in 100 ml distilled water (90°C) which was then added to a solution containing 1.89 g $NaHCO_3$ and 13.15 g NaCl, the combination being adjusted to pH 8 and made up to 225 ml with H_2O. The washed CNBr-4B and the gelatin solution were mixed together and incubated for 16 hours at 22°C. The resulting gelatin conjugated Sepharose-4B was then washed with 500 ml of 0.1 M sodium acetate, pH 4.5, followed by 500 ml of 0.1, Tris/HCL, pH 8.5. The two washes were repeated once more. The final conjugated product was suspended in a PBS/citrate buffer and stored at 4°C.

Microaffinity Assay. The gelatin-coated Sepharose 4B beads (sufficient amount to give 1 ml packed volume) were placed in a capped conically graduated centrifuge tube (15 ml, Corning No. 25310). The beads were suspended in 10 ml of the PBS/citrate buffer without sodium azide. A good suspension was obtained by vortexing the mixture for approximately 10 seconds. The suspension then was centrifuged at 4000 rpm, 4°C, for 2 minutes in a Damon IEC PR-J centrifuge. The supernatant and excess beads were then removed by aspiration with care not to remove the gel particles below the 1 ml mark on the tube.

A sample of 0.8 ml human plasma (or 0.2 ml human cryoprecipitate) was added to the gel particles and the resultant mixture was vortexed for

10 seconds and thereafter allowed to stand for two minutes. PBS/citrate buffer (10 ml) was added to the gel sample mixture, vortexed until evenly suspended and then allowed to stand an additional two minutes. The gel suspension then was centrifuged for two minutes at 4000 rpm and 4°C.

After centrifugation, the supernatant was removed by aspiration as set forth above and the gel then was washed with 10 ml of PBS/citrate by the vortex and centrifugation procedure described above. This step was repeated for a total of 4 washes. After the last wash, the supernatant was carefully removed so that the surface of the gel is almost dried and none of the gel is removed. Exactly 2 ml of 6 M urea in PBS/citrate buffer was added to the gel and vortexed until evenly suspended (approximately 10 seconds) and the sample was allowed to stand for two minutes. The resultant mixture was centrifuged for five minutes at 4°C and 4000 rpm. The supernatant then was removed with a pipette and saved.

Fibronectin concentration was determined by adding 0.2 ml of a concentrated protein dye (Bio-Rad protein dye reagent; Coomassie Brilliant Blue G-250) to 0.8 ml of the urea supernatant. After six minutes, the light absorbance of the resultant mixture at 595 nm was determined. As a comparison, a blank consisting of 0.8 ml of the buffer was processed similarly to the protein sample following all the steps of the assay. Corresponding fibronectin concentration in the original sample then was estimated by the exponential regression parameters shown under Results.

Standard Curve. A standard curve for the quantitation of fibronectin was constructed as follows. Gelatin-coated Sepharose 4B beads equilibrated

with PBS/citrate buffer were packed to 1 ml aliquots by centrifugation under the same conditions as described for the assay. Two ml of 6 M urea were added, the gel beads were resuspended by vortexing, and packed by centrifugation as per the assay. The supernatants, which contain 6 M urea diluted with the volume of PBS/citrate buffer originally present in the beads, were combined and used as the solution for preparing the fibronectin stock solution and subsequent dilutions for the standard curve. This is necessary because the concentration of urea affects the absorbance of the protein dye complex. A stock solution of 1.25 mg/ml was prepared using the freeze-dried fibronectin preparation and diluted accordingly with the above urea solution. Subsequently, 0.8 ml of the fibronectin dilutions were mixed and 0.2 ml of the protein reagent dye and the absorbance was read as per the conditions of the assay. The amounts of fibronectin present in the 0.8 ml of the standard were then plotted against the absorbance at 595 nm to obtain the standard curve.

RESULTS AND DISCUSSION

Preliminary considerations. Engvall and Ruoslahti (18) have reported that preparative affinity chromatography of plasma on gelatin coupled to Sepharose gives electrophoretically and immunologically pure fibronectin after extraction with 8 M urea. Scott et al.(19) have established that fibronectin is completely removed from plasma although some contaminant impurities are also recovered after urea elution. These two observations set the prerequisite conditions for the development of a quantitative microaffinity assay for fibronectin in plasma. The assay depends on the binding of plasma fibronectin to gelatin-coated Sepharose beads, washing of the unbound material, extraction of the bound fibronectin with urea, and quantitation of fibronectin by a colorimetric procedure (20) utilizing a commercially available protein dye reagent. Preliminary experiments attempting to scale down the preparative method by using commercially available microcolumns gave inconsistent results, probably because of poor quality control of the columns themselves, especially the bottom filter part. Therefore, a method utilizing centrifugation in conical tubes was developed and described below.

Optimum Urea Concentration. When human plasma is subjected to the microaffinity assay conditions as described under Materials and Methods, but different concentrations of urea are used for the extraction, results as shown in Figure 1 are obtained. Maximum dye absorbance is observed with 6 M urea extraction. This is due to two reasons. Increasing urea concentration interferes with the dye absorbance and lower urea concentrations

extract less protein. Thus, a balance between protein extracted and urea interference is achieved at the 6 M concentration.

Optimum Absorbance Wavelength. Human plasma fibronectin extracted from the beads with 6 M urea and reacted with the protein-dye reagent using the blank as per the assay exhibits the absorbance spectrum of Figure 2. A plateau is obtained between 575 to 595 nm which can be utilized to quantitate the protein optimally.

Standard Curve. A standard curve was prepared by using purified fibronectin as described under Materials and Methods. The relationship between absorbance at 595 nm and amount of fibronectin reacted with the dye was not linear. An exponential regression of the type

$$Y = Ae^{BX} \qquad (1)$$

was performed which produced the following values: A = 5.93, B = 4.76, and correlation coefficient of 0.9663, where Y represents the amount of fibronectin (μg) in the dye reaction mixture and X the absorbance at 595 nm.

The Y value obtained by regression has to be multiplied by 2.5 and divided by V (where V is the volume of plasma in ml applied to the beads) to obtain the amount of fibronectin in the 2 ml urea extract. The factor 2.5 derives from the fact that only 0.8 ml of the 2 ml urea supernatant are utilized for the dye assay (thus, 2/0.8 = 2.5). A further correction has to be made for the distribution of the extracted fibronectin between the urea supernatant and the urea solution remaining in the beads. For these experiments, the dilution factor (L) was 0.683, i.e., 68.3% of the fibronectin was found in the supernatant and the rest in the liquid of the

beads. In other words, although 2 ml 6 M urea are added to 1 ml of packed beads, the total amount of extracted fibronectin is distributed in a larger than 2 ml volume because of the PBS/citrate volume contained in the beads.

The dilution factor (L) was determined by preparing a bovine serum albumin solution in 6 M urea, reading the absorbance at 280 nm, adding 2 ml of this solution to 1 ml of the packed beads, vortexing and centrifuging and reading the absorbance again of the supernatant. Under these conditions, 68.3% of the original absorbance was found in the supernatant.

Taking into consideration the aforementioned, the final formula for determining the amount of fibronectin (F) in plasma in micrograms/ml is:

$$F = 2.5\ Y/VL \qquad (2)$$

with all parameters as explained above

Quantitation in Human Plasma. When different dilutions of the same plasma are made with the PBS/citrate buffer and these samples are subjected to the microaffinity assay, a linear relationship is obtained between plasma dilution and amount of fibronectin as determined colorimetrically (Figure 4). Furthermore, when various human plasma samples are processed through the assay, and the final urea extract is analyzed by SDS polyacrylamide gel electrophoresis, the results shown in Figure 5 are obtained. Fibronectin is the predominant component in the extract. Minor, probably gelatin binding peptide contaminants, constitute less than 5% of the electrophoresed protein. Thus, the assay provides a reasonable estimate of fibronectin in human plasma.

Correlation with the Immunoturbidimetric Assay. Twenty-two human plasma samples from healthy volunteers were assayed simultaneously by the

microaffinity method and by the commercially available kit for the immunoturbidimetric assay as supplied by Boehringer-Mannheim. A poor correlation (i.e., r = 0.5032) was obtained between the two assays (Figure 6). The reason for the lack of good correlation is unknown. It is possible that immunoreactivity and gelatin binding are not expressed proportionally within a sample of plasma fibronectin. Binding of plasma fibronectin by a substance which limits its immunoreactivity has been suggested after burn injury (21).

The concentration of fibronectin in CPD anticoagulated human plasma was 270± 57 ug/ml by the immunoturbidimetric method and 356± 87 ug/ml by the microaffinity assay.

Comments. The microaffinity assay described in this report provides a quantitative determination of fibronectin in human plasma which does not require the use of specific antisera, or radioisotopes. All the reagents are commercially available, and the beads can be obtained coated with gelatin from at least three sources (Bio-Rad Laboratories, Pharmacia, and Sigma). A further advantage of the method is that the assayable fibronectin exhibits gelatin binding properties which have been proposed to be involved in the opsonization of collagen covered microaggregates (6). Thus, the assay measures fibronectin which is able to bind collagen and, therefore, may have some relevance to its biological activity.

As determined in preliminary experiments, this assay is also applicable to the quantitative estimation of fibronectin in mouse, rat, rabbit, dog, and baboon plasma. Other species have not been examined as yet.

ACKNOWLEDGMENT

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The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or Naval Service at large.

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FIGURE LEGENDS

FIGURE 1 Absorbance at 595 nm of human plasma fibronectin extracted with different concentrations of urea after binding to gelatin-coated Sepharose 4B beads and reacted with the Bio-Rad dye reagent (Coomassie Blue G-250).

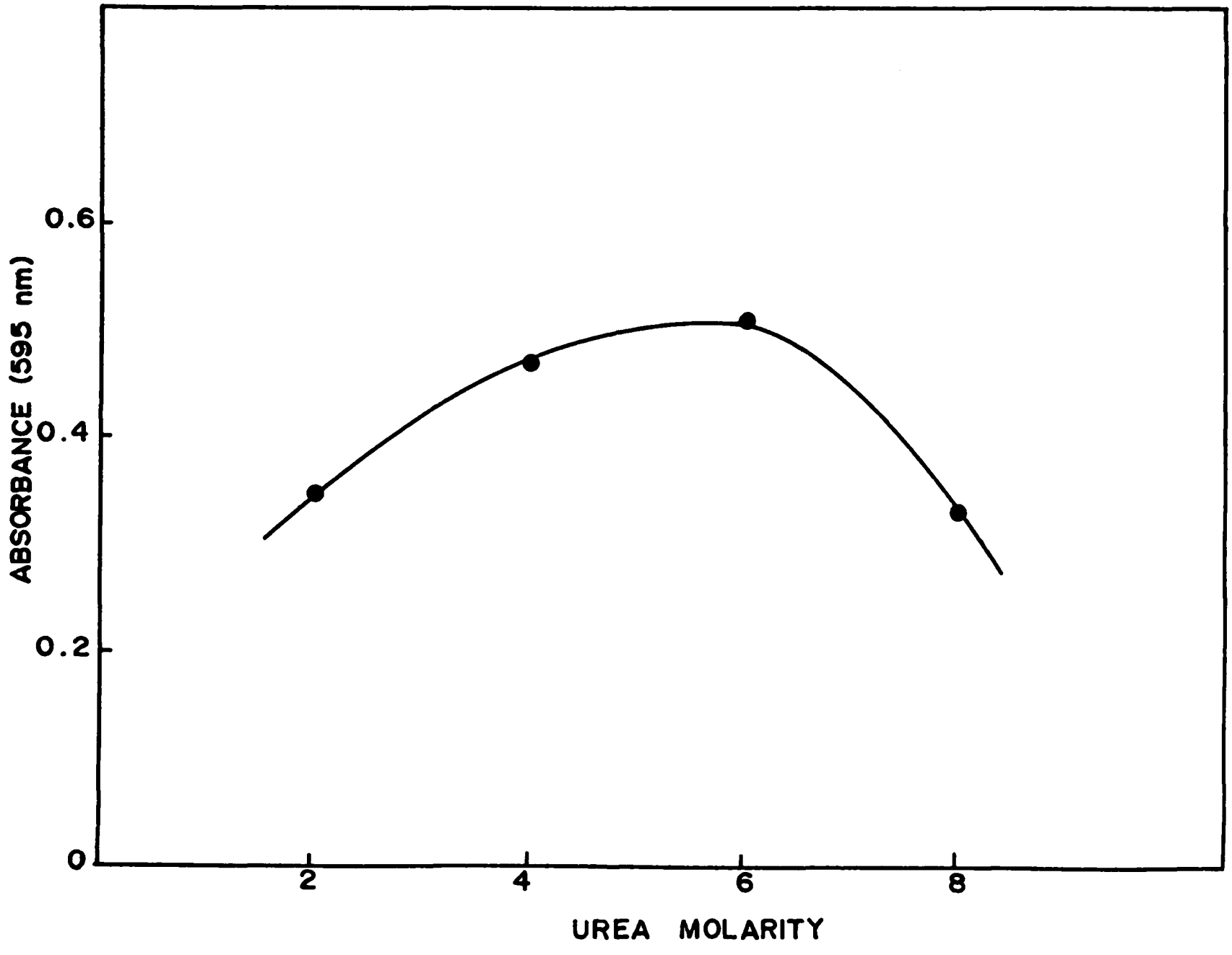
FIGURE 2 Absorbance spectrum of human plasma fibronectin extracted from the gelatin-coated Sepharose 4B beads with 6 M urea and reacted with the Bio-Rad protein dye reagent. A plateau region is obtained between 575 and 595 nm.

FIGURE 3 Absorbance at 595 nm of different amounts of isolated human plasma fibronectin reacted with the Bio-Rad protein dye reagent. An exponential regression of the type $Y = Ae^{BX}$ exhibited a correlation coefficient of 0.9663.

FIGURE 4 Linear relationship between amount of fibronectin as determined by the microaffinity assay and dilution of human plasma samples (A,B and C) subjected to the analytical procedure.

FIGURE 5 SDS polyacrylamide gel electrophoresis in 5% T gels of samples of human plasma from various healthy donors subjected to the microaffinity assay and electrophoresing the final 6 M urea extract. The major band corresponds to the fibronectin subunits.

FIGURE 6 Correlation scattergram between human plasma fibronectin determined by the microaffinity assay and by the Boehringer-Mannheim immunoturbidimetric kit. The bars show (±) one standard deviation from the mean (center of the cross). The correlation coefficient is 0.5032.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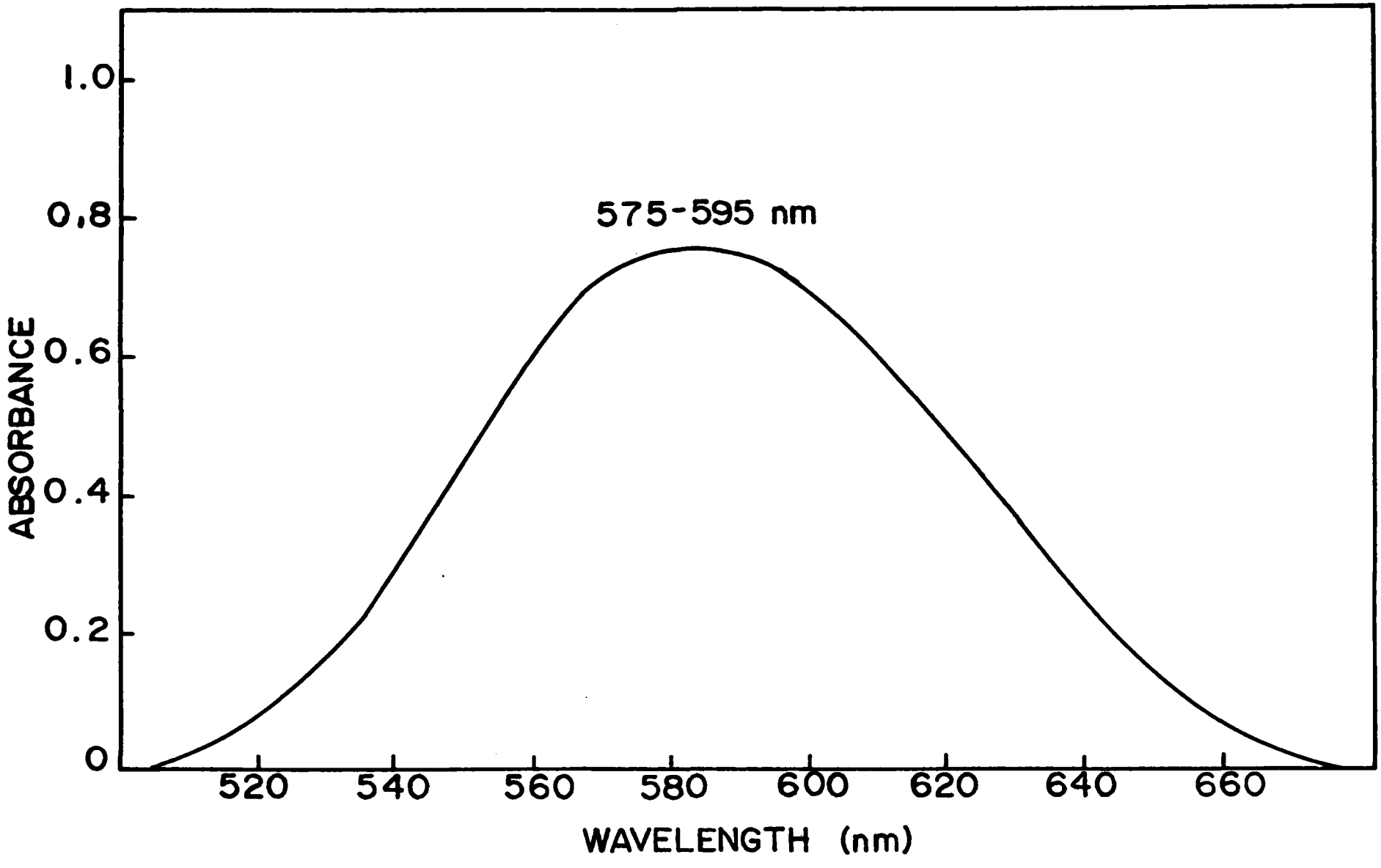
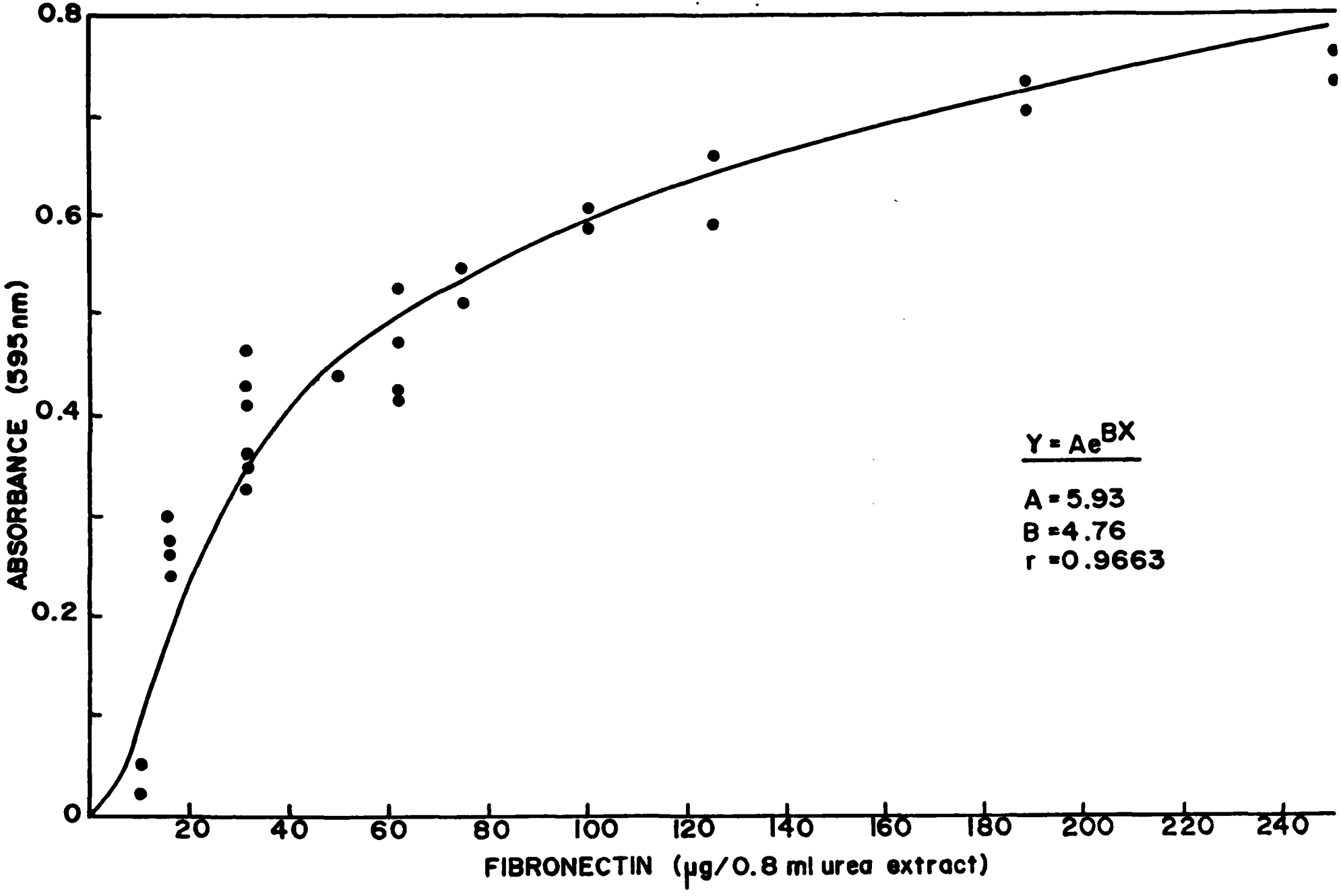
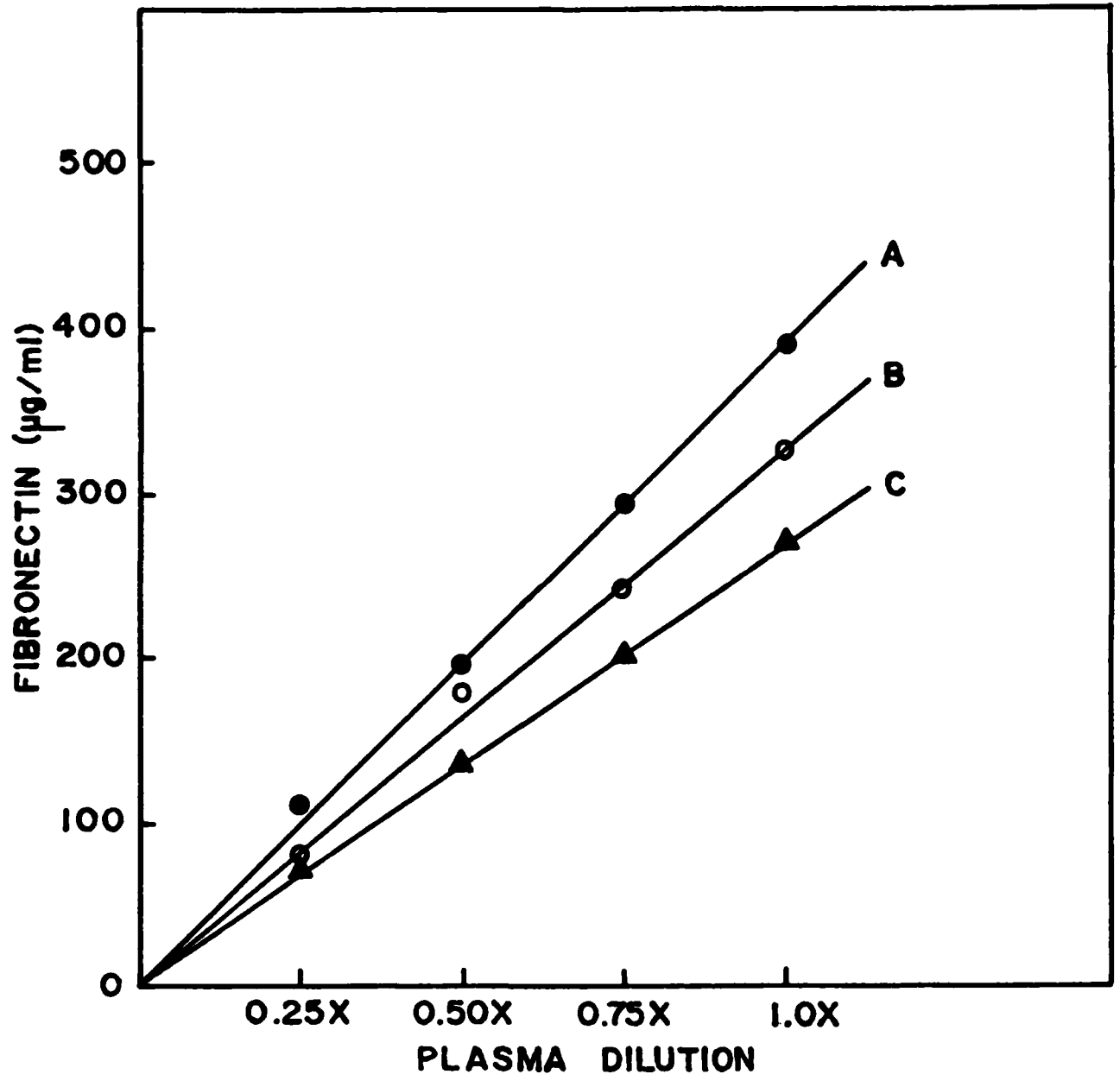
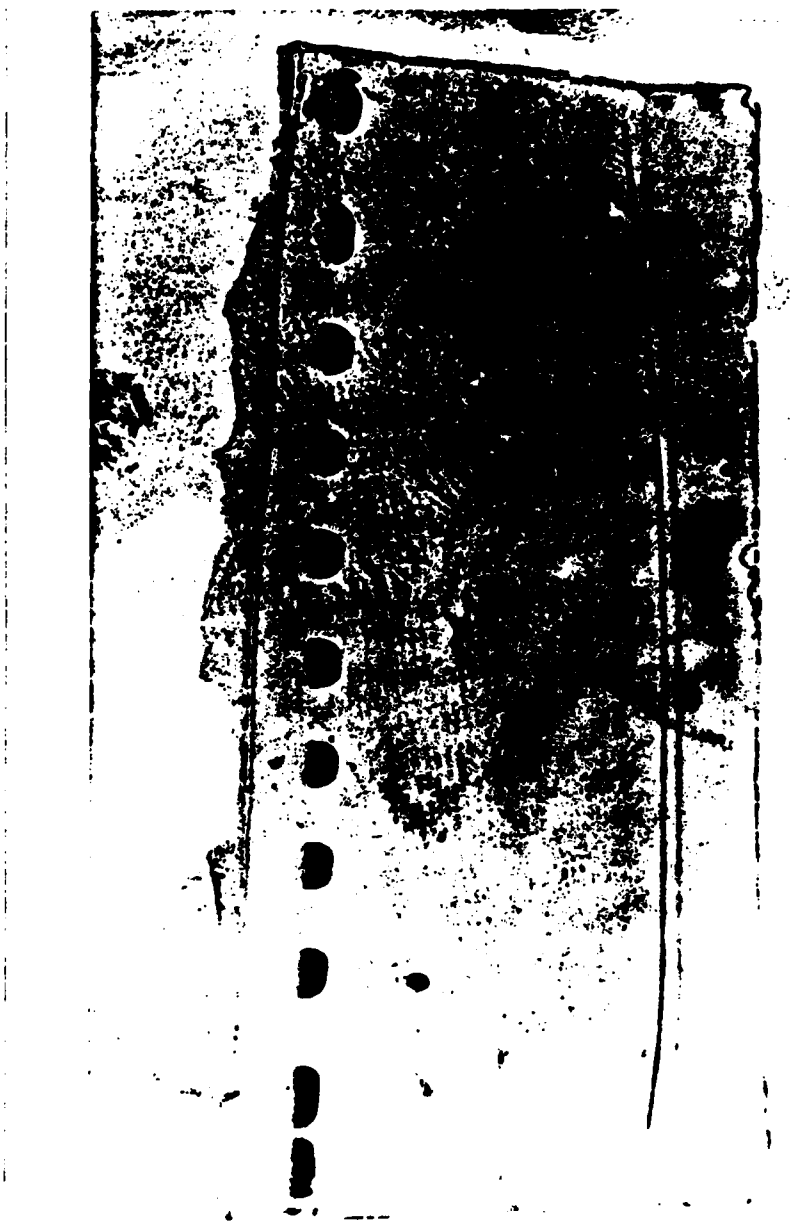
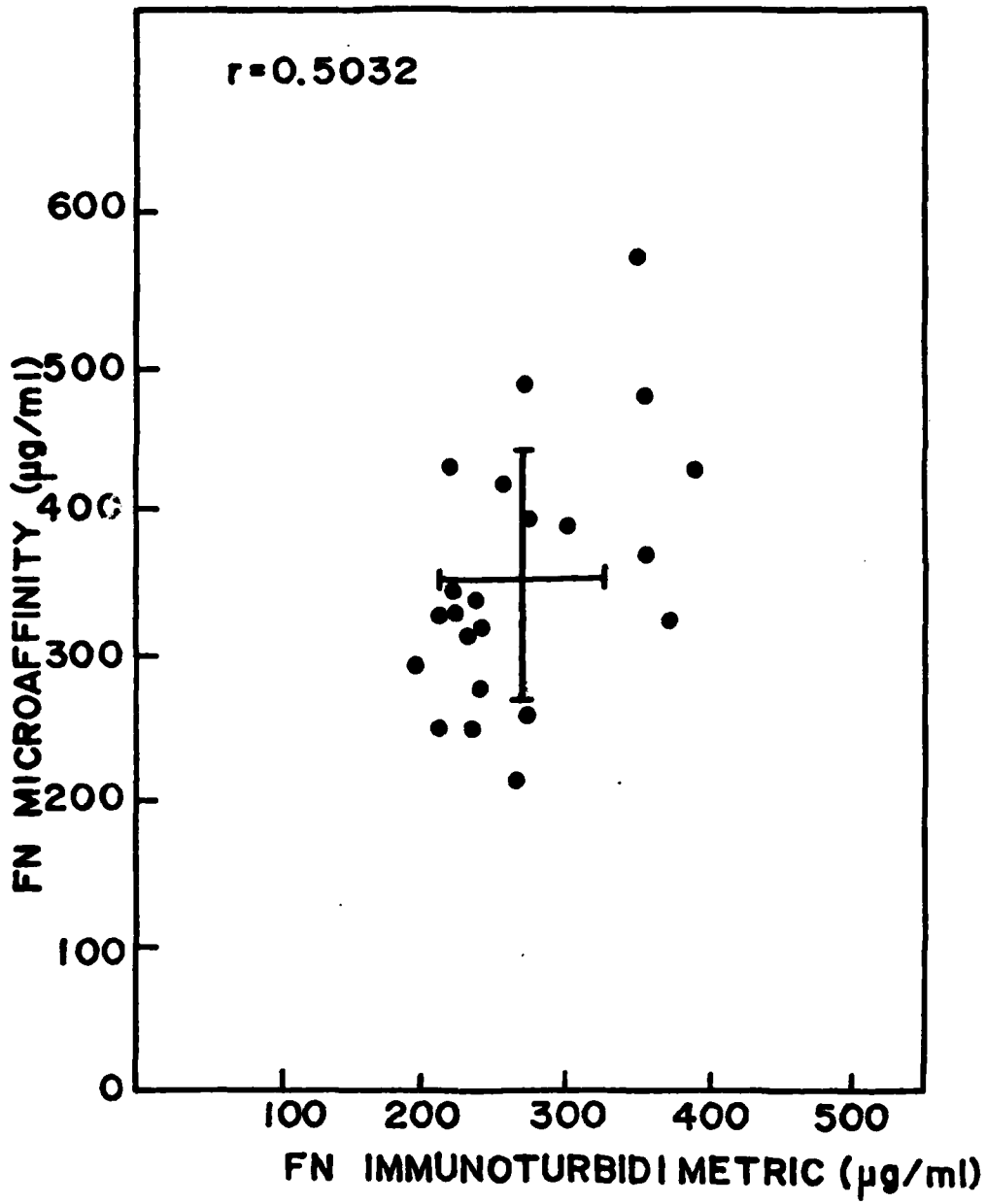
0.6
0.4
0.2
0
2
4
6
8
UREA MOLARITY


1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
575-595 nm
520
540
560
580
600
620
640
660
WAVELENGTH (nm)


0.8
0.6
0.4
0.2
0
ABSORBANCE (595nm)
20
40
60
80
100
120
140
160
180
200
220
240
FIBRONECTIN (µg/0.8 ml urea extract)
Y = Ae^BX
A = 5.93
B = 4.76
r = 0.9663


500
400
300
200
100
0
FIBRONECTIN (µg/ml)
A
B
C
0.25X
0.50X
0.75X
1.0X
PLASMA DILUTION

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600
500
400
300
200
100
0
FN MICROAFFINITY (µg/ml)
100
200
300
400
500
FN IMMUNOTURBIDIMETRIC (µg/ml)

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